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(54) Title: METHOD OF EXTRACTING NUCLEIC ACIDS FROM MICROBIAL SAMPLES

(57) Abstract: The present invention provides methods of extracting nucleic acids from environmental samples, such as terrestrial soil. The methods involve washing intact microbes from the sample such that humic acid is removed from the sample before the cells are broken and their contents released into the sample. Higher yields and more highly purified nucleic acids are obtainable from environmental samples according to the methods.

METHOD OF EXTRACTING NUCLEIC ACIDS FROM MICROBIAL SAMPLES

Field of the Invention

This invention relates to nucleic acid extraction from microbial samples.

Background of the Invention

The following discussion of the background of the invention is merely provided to aid the reader in understanding the invention and is not admitted to describe or constitute prior art to the present invention.

Soil is a valuable microbiological resource, having a very large and diverse microbial population. The isolation of bacteria from soil samples is a useful tool for studying the genes that encode important metabolic pathways, as well as for other purposes. But traditional means of cell cultivation yield only a very small proportion of the total microbial community. Methods of obtaining microbial DNA from soil samples often include bacterial cell lysis *in situ* followed by direct nucleic acid extraction. While optimizing the diversity of the recovered bacterial species, such methods have proven to generate DNA of relatively low molecular weight and/or samples of low purity. For example, genomic DNA isolated directly from soil via a Bio101 or similar extraction kit (Bio101, La Jolla, CA) is generally less than 20kb in size (Liles *et al.*, 2003). Consequently, such DNA can only be used for PCR amplification of single gene homologs or for the construction of single gene libraries (Henne *et al.*, 2000; Norris *et al.*, 2002; Rees *et al.*, 2003; Kabir *et al.*, 2003). Those libraries are not suitable for the cloning and isolation of antimicrobial small molecule pathways requiring coordinated expression of more than one gene. On the other hand, techniques which include pre-isolation of the bacterial fraction often result in poor DNA yield and low cloning efficiency (Beja *et al.*, 2000; MacNeil *et al.*, 2001). Typically, Courtois *et al.* (2003) recently reported the construction of a 5,000 cosmid clone library from an environmental soil sample with an average insert size of 50kb. To counteract the poor DNA yield obtained from pre-isolated bacterial population, researches have included an enrichment step in a variety of laboratory media (Daniel, 2002; Knietsch *et al.*, 2003a and b). While such techniques clearly amplify the bacterial population and increase the amount of DNA recovered, they also greatly affect the diversity of species represented in the enriched sample since only 0.1 to 1%

of bacterial species are able to divide and grow in laboratory media. The present application provides methods of extracting nucleic acids from environmental samples that avoids these disadvantages. Genomic DNA prepared from environmental samples according to the method of the present invention can be used to construct cosmid libraries of 10^5 to 10^6 clones per μg of DNA with an average insert size of 40kb.

Summary of the Invention

The present invention is broadly directed to methods of extracting nucleic acids from microbial samples, such as soil. The present methods involve washing intact microbes from the sample such that humic acid, phenolic compounds (e.g., polyphenol and phenol), and other contaminants are removed from the sample before the microbes are broken and their contents released into the sample.

The present invention provides in one aspect a method for extracting nucleic acids from a plurality of microbes in a sample, which method comprises (a) providing a sample comprising a plurality of microbes, a starting amount of humic acid, and other substances, wherein each of the microbes comprises one or more nucleic acids, (b) washing said microbes without substantially releasing said nucleic acids from said microbes comprising (i) washing said sample with a separation buffer wherein about 50% to about 80% of said starting amount of humic acid is removed, leaving an intermediate amount of humic acid, and (ii) subsequently washing said microbes with a wash buffer, wherein greater than 50% of said intermediate amount of humic acid is removed from said sample, wherein said washing steps are conducted in the absence of proteinase K and a lysogenic concentration of cell lysis agent, (c) opening said microbes and releasing said nucleic acids from each of said microbes, and (d) recovering said nucleic acids.

In certain embodiments, greater than 60%, 70%, 80%, 90% or 95% of said intermediate amount of humic acid is removed as a result of the washing with a wash buffer in step (b)(ii).

In various embodiments the sample is an environmental sample. The sample can be collected from lakes, ponds, beaches, stromatolites animal waste or atmospheric air. The sample can be soil. The microbes can be bacteria or fungi. The nucleic acid can be DNA.

The separation buffer is preferably slightly acetic. The pH may be about 5.0. The separation buffer may comprise a humic acid removing agent which can be acid washed PVPP or hexadecyltrimethyl-ammonium bromide (CTAB).

The wash buffer is preferably a high salt buffer and is slightly basic, e.g. between pH 7.0 and 9.0. In one embodiment, the wash buffer is at a pH of 8.0. The buffering capacity of the wash buffer can be provided by a phosphate salt or by Tris buffer (tris hydroxymethyl) aminomethane). The wash buffer may comprise CTAB or PVPP. The wash buffer may have an ionic strength of at least 1.0. In some embodiments, the wash buffer contains greater than or equal to 0.5% (w/w) CTAB. The wash buffer may comprise 1.5 M NaCl. In one embodiment, the wash buffer contains about 1% (w/w) CTAB and about 1.5 M NaCl.

In some embodiments the nucleic acid is released from the microbes by applying mechanical force to the microbes, e.g., by vortexing the microbes with solid objects. The solid objects may be beads.

In various embodiments, the recovering step can be extraction by electrophoresis using an extraction buffer. The extraction buffer can comprise a chelating agent and a humic acid removing agent. The extraction buffer may have an ionic strength of about 1.5 M to about 2.0 M. The chelating agent may be EDTA or EGTA and the humic acid removing agent may be PVPP or CTAB. The electrophoresis can be pulse field electrophoresis.

The present invention also provides a method for extracting nucleic acids from a plurality of microbes in a sample, which method comprises (a) providing a sample comprising a plurality of microbes, a starting amount of humic acid and other substances, wherein each of said microbes comprises one or more nucleic acids, (b) washing said microbes without substantially releasing said nucleic acids from said microbes comprising (i) washing said sample with a separation buffer which is slightly acetic, wherein about 50% to about 80% of said starting amount of humic acid is removed, leaving an intermediate amount of humic acid, and (ii) subsequently washing said microbes with a wash buffer containing at least 0.5% (w/w) CTAB, at a pH of about 8.0, and an ionic strength of at least 1.0, wherein greater than 50% of said intermediate amount of humic acid is removed from said sample, wherein said washing steps are conducted in the absence of proteinase K and lysogenic concentration of cell lysis agent, (c) opening said microbes and releasing said nucleic acids from each of said microbes; and (d) recovering said nucleic acids released from said microbes by subjecting said nucleic acids to pulse field electrophoresis.

The present invention further provides a method for constructing a cosmid library from the recovered nucleic acids, which method comprises (a) subjecting said

nucleic acids to one or more treating steps so as to obtain nucleic acids in a form which can be ligated to a cloning vector, (b) inserting said nucleic acids into a vector, (c) introducing said vector into a cell wherein said cell is transfected or transformed, and (d) culturing said transfected or transformed cell, wherein cosmid libraries of 10^5 to 10^6 clones per μg of the nucleic acids are constructed with an insert size ranging between 30kb and 50kb.

The summary of the invention described above is not limiting and other features and advantages of the invention will be apparent from the following detailed description of the invention, as well as from the claims.

Detailed Description of the Invention

The present invention provides methods of extracting nucleic acids from microbial samples, such as soil. Humic acids and other phenolic compounds present in samples can bind to nucleic acids, such as DNA, and be difficult to remove from the sample. These contaminants reduce the efficiency of PCR, complicate the nucleic acid purification process, and interfere with nucleic acid cloning processes that follow the nucleic acid isolation. The present methods involve washing intact microbes from the sample such that humic acid, phenolic compounds (e.g., polyphenol and phenol), and other contaminants are removed from the sample before the microbes are broken and their contents released into the sample.

"Humic acid" refers to allomelanins found in soils, coals, and peat, and results from the decomposition of organic matter, particularly dead plants. They are a mixture of complex macromolecules having polymeric phenolic structures with the ability to chelate metals, especially iron. These polymers can have a few to a several hundred phenols linked together, and sometimes occur in a branched conformation.

Without wanting to be bound by any particular theory, it is believed by Applicant that these contaminants bind to nucleic acids, and inhibit nucleic acid cloning. It is also believed that these contaminants may inhibit other nucleic acid manipulations that occur later in the nucleic acid extraction and purification process. Thus, by removing these contaminants early in the nucleic acid extraction and manipulation process, and prior to releasing the nucleic acid into the sample, it is believed that many troublesome substances are removed that would otherwise bind to the nucleic acid released from the cells, complicate the extraction process, and result

in poor yields. Many of these substances bind tightly to nucleic acids and are extremely difficult to remove once bound.

The present invention provides methods to remove humic acid from the sample. For example, buffers in which humic acid is soluble can be useful for this purpose. A soil or other environmental sample can be washed in such a buffer and the buffer separated from the microbial portion of the sample. This process can be repeated two, three, four, or more times if necessary. But it is highly preferable that at least a majority of the humic acid, and more preferably as much as possible (e.g., 60% or 70% or 80% or 90% or 95% or more of the humic acid), be removed from the sample before opening the microbes. Humic acid is soluble in many aqueous buffers, and any are useful in the present invention.

A "sample" is a portion or aliquot of matter from which nucleic acids are to be extracted. The present methods are applicable to a variety of environmental samples. "Environmental samples" include, but are not limited to, samples having their source in the environment, including samples from lakes, ponds, beaches, terrestrial soil samples, stromatolites, animal waste, and even samples produced by filtering atmospheric air. Other examples of environmental samples include feces and marine samples (e.g., open water sediments and samples from marine animal gut).

By "washing the sample" with a buffer is meant that the sample is solubilized, suspended, or rinsed with the buffer and the portion containing microbes or nucleic acids isolated or separated from another portion. Usually, the portion containing the microbes or nucleic acids will be separated from the buffer by forcing it into a solid phase (e.g., by centrifugation).

By "washing the microbes" is meant that the microbes are contacted with a solution and suspended in the solution. The microbes are later separated from the solution or from a part of the solution. In some embodiments, the separation is performed by centrifugation and the cells can be resuspended in fresh buffer. Thus the washing step removes one or more components from the environment of the microbes.

The methods include the use of a separation buffer, a wash buffer and a nucleic acid extraction buffer. By "buffering capacity" is meant the ability to maintain a constant pH despite the addition of basic or acidic compounds.

The separation buffer is slightly acidic (e.g., pH of about 5.0) and serves to separate the microbes from the other substances of the sample such as soil particles

and other live cells. The separation buffer also serves to trap a part of the humic acids present in the sample (e.g., soil). The slightly acetic pH assists in dispersing soil particles. The buffer may contain acid-washed PVPP, CTAB or any other agent that can trap humic acids and other phenolic compounds. In one embodiment, the buffer contains 0.1 M sodium phosphate, pH 5.0, and 1g/L acid-washed PVPP. In another embodiment, the buffer comprises CTAB. Persons of ordinary skill in the art will realize that other buffers may be substituted. These buffers are useful in suspending soil particles from the sample and separating microbes that are bound to soil particles. The washing with a separation buffer can remove about 50% to about 80% of the starting amount of humic acid.

Subsequent to the washing with the separation buffer, the sample, for example a terrestrial soil sample, is washed with a wash buffer before the microbes are broken and opened. The wash buffer removes most of the remaining humic acids, phenol and other phenolic compounds, various salts, and other contaminants (including unknown contaminants) before the nucleic acids are released from the microbes and thereby exposed to these contaminants in the sample. Once bound to nucleic acids, humic acids are difficult to remove. Thus it is important that the microbial cells are not lysed and that the nucleic acids are not released upon the washing step(s).

In various embodiments the wash buffer of the invention is slightly basic. By "slightly basic" is meant that the pH is between 7.0 and 9.0. In various embodiments the wash buffer contains a high concentration of salt, which is useful for removing ions (e.g., Hg^{2+}, which interferes with DNA cloning). The high salt concentration is also useful for preventing DNA from binding to soil particles by occupying the potential binding sites on the soil particles. By a "high salt buffer" is meant a buffer with an ionic strength of at least 1.0. In other embodiments, the buffer has an ionic strength of about 1.5 M to about 2.0 M. In certain embodiments, the wash buffer contains 1.5 M NaCl. In certain embodiments the sample is washed in a buffer containing about 1% (w/w) hexadecyltrimethyl-ammonium bromide (CTAB). In one embodiment, the buffer contains 1.5 M NaCl, 1% hexadecyltrimethyl-ammonium bromide (CTAB), 0.2 M Na_3PO_4 buffer, pH 8.0. In another embodiment, the buffer contains 1.5 M NaCl, 0.1 M Tris, 0.1 M EDTA, 1% hexadecyltrimethyl-ammonium bromide (CTAB), pH 8.0. These buffers are useful for solubilizing humic acid, ions, and other water soluble contaminants.

A high salt buffer containing hexadecyltrimethyl-ammonium bromide (CTAB) is useful as a wash buffer since a small quantity of the buffer's constituents are likely to remain until the microbes are opened, thereby exposing the microbial nucleic acids to the buffer constituents. It is also noted that some quantity of humic acid will remain in the sample after the removal of a majority of the humic acid. Thus, some quantity of humic acid may still be present when the microbial cells are opened. Without wanting to be bound by any particular theory, it is believed by the Applicant that the high salt concentration and the presence of CTAB in the buffer will inhibit remaining humic acid from binding irreversibly to nucleic acids, thereby complicating later manipulations of the DNA. These constituents will also inhibit the nucleic acids from binding to remaining soil particles.

Proteinase K is an endolytic serine protease that cleaves peptide bonds at the carboxylic sides of aliphatic, aromatic or hydrophobic amino acids. By "detergent" is meant any of the molecules that reduce the surface tension of water and have both a hydrophobic moiety and a hydrophilic moiety. Sodium dodecyl sulfate and Triton X 100 are examples of detergents and surfactants, although the person of ordinary skill will realize many detergents and surfactants that are used in scientific processes, and those detergents are contemplated within this definition. Buffers containing these components are widely used in extraction and bacterial lysis applications. These components have the effect of encouraging the lysis or opening of the bacteria. But the present invention involves removing humic acid and other contaminants prior to opening the microbes and prior to exposing the nucleic acid being extracted from the microbes to the buffer components. Thus, it is preferable that buffers containing detergents at an amount or strength that can encourage the lysis or opening of the microbes not be used in the wash steps of the present invention, although they may be used in later steps when it is desirable to open the microbes. However, mild detergents such as CTAB which do not contribute to the lysis or opening of the microbes can be added in the separation or wash buffers.

The separation buffer and wash buffer do not contain any cell lysis agent present at a lysogenic concentration, e.g., lysozyme or other enzymes or agents that promote lysis of cells. A "cell lysis agent" is a compound that promotes the lysis of cells. A lysogenic concentration is that amount that will cause at least 10% of the cells to lyse.

Washing the microbes in wash buffers can dissolve humic acids and remove at least 50% of the humic acids left over after the wash with a separation buffer, or at least 60% or at least 70% or at least 80% or at least 90% or at least 95%.

An "extraction buffer" is a buffer designed to facilitate the retrieval of nucleic acids (e.g., DNA) from microbial samples. The nucleic acid extraction buffer serves as a medium where the nucleic acids can be released and is therefore designed to prevent nucleic acid degradation and contamination. The extraction buffer serves to solubilize nucleic acids released from the microbial cells. It contains one or more chelating agents (e.g., EDTA, EGTA) that inhibit DNases by trapping ions required for their function. The extraction buffer also contains humic acid removing agent such as CTAB or PVPP. The pH of the extraction buffer is close to neutral. In some embodiments, the extraction buffer has an ionic strength of about 1.5 M to about 2.0 M. In one embodiment, the buffer contains 1.5 M NaCl, 1% hexadecyltrimethyl-ammonium bromide (CTAB), 0.1M EDTA and 0.2 M Na_3PO_4 buffer, pH 8.0.

The term "about" as used herein means ± 5%.

A buffer in which humic acid is "soluble" means that humic acid has a solubility in the buffer of at least 0.5 g/L, more preferably at least 1.0 g/L, and most preferably at least 2.0 g/L.

By "opening the microbes" is meant that the microbes' cell membranes are disrupted or burst and the contents of the microbe are spilled into the medium. A release of nucleic acids by the microbes is "substantial" when more than 10% of a sample of control microbes are disrupted or burst and release their nucleic acid into the medium under identical conditions.

Opening the microbes can be measured by direct counting. The following is one procedure that can be used to count the microbes: A portion (e. g. 1/10) of the sample is suspended in 1 ml of buffer (0.1M Tris, 10 mM EDTA) and vortexed for 15 seconds. 1.5 ul of the suspension was mixed with 1.5 ul of DAPI solution (4',6-diamidino-2-phenylindole, 0.05 mg/ml in water) on a poly-L-lysine covered slide. The total number of intact cells is determined by counting the DAPI-stained blue fluorescent cells under a fluorescent microscope, with an appropriate filter combination for DAPI. Appropriate dilution factors were used to convert the counting results to intact cells in the sample. The percentage of cells that have been opened can then be calculated based on the difference between the cell count before and after the treatment.

By "extracting nucleic acid from the microbes" is meant that any type of DNA and/or RNA having its source in the microbes is isolated from the microbes.

Another feature of the invention is the use of electrophoresis to purify the nucleic acid. In one embodiment, the electrophoresis is pulse field electrophoresis. Due to the physical and chemical nature of humic acid, traditional methods of separating it from DNA such as gel filtration, extraction, or even conventional electrophoresis are less effective for removing this contaminant. The present inventors discovered unexpectedly that the methods described herein are highly effective for removing these contaminants and significantly enhance the quality and quantity of DNA yields obtained from environmental samples.

The following examples illustrate various aspects of the invention, but in no way are intended to limit the scope thereof.

Example 1

This example presents one embodiment of the present invention. In this example the environmental sample is a soil sample, which is treated by washing the intact microbes from the environmental sample under circumstances that remove humic acid and phenol from the sample without substantially releasing nucleic acid from the microbes into the sample. The microbes are then opened and the nucleic acid is released after the washing step. The sample is then subjected to pulse field electrophoresis to extract and purify the nucleic acid.

1. 100g of soil sample and 150 ml of 0.1M sodium phosphate buffer (pH 5.0) containing 0.1g/L acid-washed PVPP (separation buffer) was combined and placed in a blender. The sample was whipped in the blender for 1 minute and then placed on ice for 2 additional minutes to remove heat generated by the blending. This step was repeated two more times to ensure that bacteria are separated from soil particles at high yield.

Acid washed PVPP is insoluble in aqueous buffers and is effective for removing humic acid. The acid washed PVPP was prepared as follows: 300g of PVPP was mixed with 4 liters of 3 M HCl at room temperature and allowed to mix for 16 hours. The PVPP was then pelleted and washed with 0.1M sodium phosphate buffer to produce the acid-washed PVPP, which is then ready for use.

2. The liquid was equally distributed between two centrifuge bottles and centrifuged at 2,000 rpm for 10 minutes at 10°C to remove large soil particles.

3. The supernatant, which contains the bacteria, was removed and placed in a clean centrifuge bottle.

4. 75 ml of 0.1 M sodium phosphate buffer (pH 5.0) was added to each bottle containing the soil pellet, and the bottles were shaken to resuspend the pellets and the samples placed back into the blender.

5. Steps 2-4 were repeated two more times to maximize the yield of bacteria from the soil particles.

6. To pellet the bacteria, the bottles containing the supernatant were centrifuged at high speed (8,000 rpm) for 10 minutes at 10 °C, and the supernatant removed.

7. The bacterial pellet was washed with DNA wash buffer (0.1 M Tris, 1.5M NaCl; 1% hexadecylmethylammonium bromide (CTAB), 0.1 M Na_3PO_4 buffer, 0.1 M EDTA, pH 8.0 ("Phosphate-CTAB buffer") at room temperature or at 37° C from 1 hour to overnight depending on the level of contamination with humic acid, which can be inferred from the intensity of the brown color in the pellet. This step is important for removing a large quantity of humic acids from the sample (more than 50%) and CTAB serves to trap those phenolic compounds. For highly contaminated samples this step may be repeated several times as required.

8. 10 ml of DNA extraction buffer (Phosphate-CTAB) was added (1.5 M NaCl, 1% CTAB, 0.1 M EDTA, 0.2 M Na_3PO_4 buffer, pH 8.0) to 5g (wet weight) of bacterial fraction, which was obtained from the pellet wash of the previous step. Alternatively, 0.1M Tris, 0.1 M EDTA, 1.5 M NaCl, 1% CTAB, pH 8.0 and many other buffers can also be used instead of the phosphate buffer, with or without the CTAB. EDTA is an essential component in this buffer because it chelates ions that are required for most DNAse degradation activity.

9. 2 g of glass beads were added to the bottle, and the sample was vortexed briefly (15 seconds) to disrupt the bacterial cells. Repetitive freeze and thaw cycles may also be used for this purpose.

10. 80 µl of proteinase K (20 mg/ml) was added and the sample incubated at 37 °C for 30 min to 1 hour to facilitate disruption of the bacterial cells and also to digest contaminating proteins.

11. 1.5 ml 20% SDS was added, the sample mixed and incubated at 70° C for 1 hour, with mixing accomplished by inverting the tube about every 15 min. This

step further completes the disruption of the bacterial cells and releases bacterial DNA into the medium.

12. The sample was then centrifuged at 3000 rpm for 15 min, with the supernatant (which contains the DNA) retained for later use. The pellet, which contains cell debris and remaining soil particles, was discarded.

13. 0.53 volumes of isopropanol was added to the supernatant to precipitate the DNA.

14. If a DNA pellet is formed at this step, the clump is scooped up with a glass pipette. If no obvious DNA pellet is formed, the sample is centrifuged at 4000 rpm for 20 min to recover the pellet.

15. The DNA pellet was washed twice with 70% ethanol to remove any salts that co-precipitated with the DNA, and dissolved in 1 ml of Tris-EDTA buffer (10 mM Tris, 1 mM EDTA, pH 8.0).

16. If the color of the DNA solution was still dark brown at this step, the sample was extracted once with phenol and twice with chloroform:iso-amyl-alcohol (24:1) to remove additional humic acid.

17. 1/10 volume of 3M sodium acetate was added and the DNA in the sample precipitated with 0.6 volumes of isopropanol to remove additional humic acid that may still be present.

18. The pellet was resuspended in 1 ml of Tris-EDTA buffer, as above.

19. The DNA sample was further purified by pulse field electrophoresis in a low melting agarose gel (1%), using a 5 kb ladder as a marker (Electrophoresis parameters: 4 V/cm, switch time 1-6 seconds, running time: 3 hours, temperature 14°C). This step serves to remove remaining humic acid and other contaminants that may be present from the DNA, as serving to size fractionate the DNA. Many instruments for conducting pulse field electrophoresis are known in the art and can be used for this purpose, for example, CHEF-DR II™ by Bio-Rad Laboratories (Hercules, CA) or equivalent. The electrophoresis buffer used was 0.5x or 1x TAE (0.04 M Tris-Acetate, 0.001M EDTA pH 8.0). Alternatively, 0.5x or 1x TBE (45 mM Tris, 45 mM Borate, 1 mM EDTA, pH 8.3) can also be used.

20. The resulting gel was stained with ethidium bromide for 30 min (5 µg/ml Et-Br in water), and de-stained for 45 min in water.

21. Gel slices containing DNA were cut from the gel under UV-light, and the DNA extracted from the gel. Various enzymes and kits are commercially

available that are suitable for purifying DNA from agarose gel. For example, the GELase™ kit from Epicentre (Madison, WI) can be used, as can NaI solution. These kits or reagents can dissolve or digest the gel and release the DNA into solution. The DNA is then concentrated or purified according to conventional methods and the manufacturer's instructions.

22. The DNA is now ready for further analysis, such as cutting with restriction enzymes, PCR, and cloning into a vector.

The person of ordinary skill in the art will also realize that various methods are available for opening the microbes. For example, freezing and thawing repeatedly combined with proteinaseK and/or lysozyme can also open bacterial cells. And variations can be made in the constituents of the buffer while achieving the same result. These various methods are also contemplated in the present invention.

Example 2

As described above, many buffers are available for removing the humic acid from the sample. Contaminants other than humic acid will also be present in environmental samples, and very often the nature of these contaminants will be unknown. The following examples present alternative buffers that may be used in the wash step (step #7 above) to remove contaminants. Persons of ordinary skill in the art will also realize many other buffers that will also be useful, and are also contemplated as within the scope of this invention. All of the buffers described here are also effective for removing humic acid and other phenolic contaminants.

In this example, the sample is treated as described above, except that 0.33 M Tris-HCl, 0.1 M EDTA, pH 8.0 is used to wash the sample instead of the Phosphate-CTAB buffer. Tris-HCl at such concentration is effective for solubilizing humic acids and removing them from the sample. In various embodiments, CTAB (e.g., 1% w/w) can be optionally present in the buffer.

Example 3

In this example, the procedures described in Example 1 were followed, except that 100 mM potassium phosphate (pH 8.0), 1.5 M NaCl, 0.2 M EDTA buffer was used instead of the Phosphate-CTAB buffer. This buffer is also effective for removing humic acid and other contaminants from the sample. CTAB (e.g., at 1% w/w) is optionally present in the buffer.

Example 4

In this example, the procedures described in Example 1 are followed except that a low osmolarity and high PVPP wash buffer containing 1.5 mM K_2HPO_4, 2 mM NaCl, 0.2 M sodium ascorbate, 0.01 M EDTA, and 2- 8% PVPP (polyvinylpolypyrrolidone) is used to remove the humic acid instead of the phosphate-CTAB buffer. This buffer also serves to dissolve and remove the humic acid and other contaminants.

Example 5

In this example, the procedures described in Example 1 were followed except that a buffer containing 100 mM Tris-HCl (pH 8.0), 1.5 M NaCl, 0.01 M EDTA, and 2-8% PVPP was used to dissolve and remove the humic acid or other contaminants instead of the Phosphate-CTAB buffer. In an alternative embodiment, 100 mM sodium phosphate (pH 8.0), 0.2 mM EDTA and 2%-8% PVPP can also be utilized.

Example 6

Stromatolites are calcium carbonate formations that are found in salt lakes. They are built by bacteria and contain diverse and layered bacterial populations. The following procedure was used to extract DNA from stromatolites according to the present invention.

Samples (25-40 g each) were collected in Storr's Lake, San Salvador, Bahamas and stored at -80C. The samples were thawed and ground with mortar and pestle. 50 ml of DNA extraction buffer was added (1.5M NaCl; 1% CTAB; 0.2 M Na3PO4 buffer, pH 8.0 or 0.1M Tris, pH 8.0, 0.1M EDTA) and the sample brought to pH 6.0 with acetic acid (about 1ml AcOH 0.2N per 20ml buffer). The sample was shaken for 30 min or more at 37 C, and filtered through a cheese cloth. Stromatolite particles retained on the cloth can undergo a second (and third) extraction process. The filtrate was centrifuged at 5000 rpm for 10 min. and the pellet resuspended in 10 ml of DNA extraction buffer. 2 g of glass beads were added and the sample shaken gently for 1 minute. 80 ul of proteinase K (20 mg/ml) was added, and the sample shaken gently at 37 C for 30 min. 3 ml 10% SDS was added and the sample incubated at 60 C for 1 hour with gentle shaking. The sample was centrifuged at 5000 rpm for 10 min and the supernatant collected. 2 ml of protein precipitation solution (Promega™,

Madison, WI) was added and the sample kept on ice for 5 minutes. The sample was then centrifuged at 10,000 rpm for 15 min and the supernatant retained and extracted once with chloroform. The sample was precipitated with 0.54 volumes of iso-propanol and dissolved in 200 ul of Tris-EDTA buffer. The sample is now ready for further DNA manipulation processes.

Example 7

In this example, DNA prepared as described in Example 1 was used for cloning into the pCC1Fos vector from Epicentre (Cat No CCFOS110). DNA from Step 21 was extracted from the gel in order to selectively recover fragments which were 30-40kb in size. One to 10μg of fragments were end-repaired using the end-repair enzyme mix from Epicentre under the conditions recommended by the manufacturer. The fragments were ethanol-precipitated in the presence 0.1vol. NaAc and resuspended in a small volume of water. At this stage, the more concentrated the DNA, the better the cloning efficiency. The DNA was ligated to the pCC1FOS vector at a ratio of 0.5μg of vector to 0.5-1.0 μg of insert and incubated overnight at room temperature. The ligation mixture was packaged *in vitro* using Lambda packaging extracts from Epicentre and transfected into *E. coli* strain EPI300 (other *E.coli, Salmonella* or *Klebsiella* strains known to be lambda-sensitive proved to be suitable hosts at this step and produced similar cloning efficiencies). A small aliquot of the transfected cells was plated on selective media in order to titer the environmental library. We routinely obtained 10^5 to 10^6 clones per μg of environmental DNA using the procedure described here.

Example 8

This example illustrates the screening of a library obtained from environmental DNA of the present invention for the production of a small bioactive molecule.

Primary screening for bioactive compounds

Libraries constructed in pCC1FOS were pre-amplified in *E. coli* or in *Klebsiella oxytoca* by plating 5,000 to 10,000 clones per plate on LB solid medium supplemented with the appropriate antibiotic and growing overnight at 30°C. Colonies (up to 5 million per library) were scraped off the plates, pooled together, and stored at -80°C after the titer was determined.

When screening for antibacterial compounds produced by the environmental clones, libraries were plated on solid LB media containing the appropriate antibiotic, and copy control inducer in order to increase the fosmid copy number obtain higher expression levels of the incoming foreign DNA. Clones were plated at a density of 500 to 800 per 100 mm Petri dish to allow for isolated colonies to form for two days at 30°C. Each plate was then subjected to UV radiation for 2 minutes to kill bacteria present on the surface of each colony and prevent them from interfering with subsequent steps. Each plate was overlayed with 6 ml of soft LB agar (0.7% agar) inoculated with 10^6 cells/ml of an indicator species such as *Staphylococcus aureus* (antibacterial screening) or *Saccharomyces cerevisiae* (antifungal screening). Plates were incubated overnight at 37°C and screened for clearing zones in the overlay around clones which produce a compound that kills the indicator strain. The average rate of confirmed hits which reproducibly prevent growth the indicator strain was 1/28,000 for *B. subtilis* and 1/50,000 for *S. cerevisiae*.

The invention illustratively described herein may be practiced in the absence of any element or elements, limitation or limitations which is not specifically disclosed herein. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention that in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the appended claims.

The contents of the articles, patents, and patent applications, and all other documents and electronically available information mentioned or cited herein, are hereby incorporated by reference in their entirety to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference. Applicants reserve the right to physically incorporate into this application any and all materials and information from any such articles, patents, patent applications, or other documents.

The inventions illustratively described herein may suitably be practiced in the absence of any element or elements, limitation or limitations, not specifically disclosed herein. Thus, for example, the terms "comprising", "including," containing", *etc.* shall be read expansively and without limitation. Additionally, the terms and expressions employed herein have been used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the inventions embodied therein herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention.

The invention has been described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form part of the invention. This includes the generic description of the invention with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

In addition, where features or aspects of the invention are described in terms of Markush groups, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group.

Other embodiments are set forth within the following claims.

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Claims

1. A method for extracting nucleic acids from a plurality of microbes in a sample, which method comprises the following steps performed sequentially:

(a) providing a sample comprising a plurality of microbes, a starting amount of humic acid and other substances, wherein each of said microbes comprises one or more nucleic acids;

(b) washing said microbes without substantially releasing said nucleic acids from said microbes comprising (i) washing said sample with a separation buffer wherein about 50% to about 80% of said starting amount of humic acid is removed, leaving an intermediate amount of humic acid, and (ii) subsequently washing said microbes with a wash buffer, wherein greater than 50% of said intermediate amount of humic acid is removed from said sample, wherein said washing steps are conducted in the absence of proteinase K and a lysogenic concentration of cell lysis agent;

(c) opening said microbes and releasing said nucleic acids from each of said microbes; and

(d) recovering said nucleic acids.

2. The method of claim 1, wherein greater than 60%, 70%, 80%, 90% or 95% of said intermediate amount of humic acid is removed as a result of the washing with said wash buffer.

3. The method of claim 2, wherein greater than 90% of said intermediate amount of humic acid is removed.

4. The method of claim 3, wherein greater than 95% of said intermediate amount of humic acid is removed.

5. The method of claim 1 wherein said sample is an environmental sample.

6. The method of claim 5 wherein said sample is from lakes, ponds, beaches, stromatolites, animal waste or atmospheric air.

7. The method of claim 5 wherein said sample is a soil sample.

8. The method of claim 5 wherein said sample is a terrestrial sample.

9. The method of claim 1 wherein said microbes are bacteria.

10. The method of claim 1 wherein said microbes are fungi.

11. The method of claim 1 wherein said nucleic acids are DNAs.

12. The method of claim 1 wherein said separation buffer is slightly acidic.

13. The method of claim 12 wherein said separation buffer is at a pH of about 5.0.

14. The method of claim 13 wherein said separation buffer comprises acid washed PVPP or hexadecyltrimethyl-ammonium bromide (CTAB).

15. The method of claim 1 wherein said wash buffer is a high salt buffer.

16. The method of claim 15 wherein said wash buffer is phosphate salt or Tris buffer.

17. The method of claim 15 wherein said wash buffer is slightly basic.

18. The method of claim 17 wherein said wash buffer is at a pH of between pH 7.0 and 9.0.

19. The method of claim 18 wherein said wash buffer is at a pH of about 8.0.

20. The method of claim 15 wherein said wash buffer comprises CTAB or PVPP.

21. The method of claim 20 wherein said CTAB is present in said wash buffer in an amount greater than or equal to 0.5% (w/w) CTAB.

22. The method of claim 21 wherein said CTAB is present in said wash buffer in an amount of about 1% (w/w) CTAB.

23. The method of claim 1 wherein said wash buffer has an ionic strength of at least 1.0.

24. The method of claim 23 wherein said wash buffer comprises 1.5 M NaCl.

25. The method of claim 1 wherein said microbes are opened and said nucleic acids are released from said microbes by applying mechanical force to said microbes.

26. The method of claim 25 wherein said mechanical force is applied by vortexting said microbes with solid objects.

27. The method of claim 26 wherein said solid objects are beads.

28. The method of claim 1 wherein said recovering in step (d) comprises purification and size fractionation of said nucleic acids.

29. The method of claim 1 wherein said recovering in step (d) is extraction by electrophoresis using an extraction buffer.

30. The method of claim 29 wherein said extraction buffer comprises a chelating agent and a humic acid removing agent.

31. The method of claim 30 wherein said extraction buffer has an ionic strength of about 1.5 M to about 2.0 M, and wherein said chelating agent is EDTA or EGTA and said humic acid removing agent is PVPP or CTAB.

32. The method of claim 29 wherein said electrophoresis is pulse field electrophoresis.

33. A method for extracting nucleic acids from a plurality of microbes in a sample, which method comprises the following steps performed sequentially:

(a) providing a sample comprising a plurality of microbes, a starting amount of humic acid and other substances, wherein each of said microbes comprises one or more nucleic acids;

(b) washing said microbes without substantially releasing said nucleic acids from said microbes comprising (i) washing said sample with a separation buffer which is slightly acidic, wherein about 50% to about 80% of said starting amount of humic acid is removed, leaving an intermediate amount of humic acid, and (ii) subsequently washing said microbes with a wash buffer containing at least 0.5% (w/w) CTAB, at a pH of about 8.0, and an ionic strength of at least 1.0, wherein greater than 50% of said intermediate amount of humic acid is removed from said sample, wherein said washing steps are conducted in the absence of proteinase K and lysogenic concentration of cell lysis agent;

(c) opening said microbes and releasing said nucleic acids from each of said microbes; and

(d) recovering said nucleic acids released from said microbes by subjecting said nucleic acids to pulse field electrophoresis.

34. The method of claims 1 or 33, wherein said method further comprises the steps of constructing a cosmid library from the recovered nucleic acids of step (d), which method comprises the successive steps of

(a) subjecting said nucleic acids to one or more treating steps so as to obtain nucleic acids in a form which can be ligated to a cloning vector;

(b) inserting said nucleic acids into a vector;

(c) introducing said vector into a cell wherein said cell is transfected or transformed; and

(d) culturing said transfected or transformed cell;

wherein cosmid libraries of 10^5 to 10^6 clones per μg of said nucleic acids are constructed with an insert size ranging between 30kb and 50kb.

35. The method of claim 34, wherein said treating steps of step (a) are size fractionation, end-repair or partial enzymatic digestion.

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(54) Title: METHOD OF EXTRACTING NUCLEIC ACIDS FROM MICROBIAL SAMPLES

(57) Abstract: The present invention provides methods of extracting nucleic acids from environmental samples, such as terrestrial soil. The methods involve washing intact microbes from the sample such that humic acid is removed from the sample before the cells are broken and their contents released into the sample. Higher yields and more highly purified nucleic acids are obtainable from environmental samples according to the methods.

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B. FIELDS SEARCHED

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Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	TEBBE, C.C. Interference of Humic Acids and DNA Extracted Directly form Soil in Detection and Transformation of Recombinant DNA from Bacteria and a Yeast. August 1993, vol. 59, No. 8, pages 2657-2665	1-35

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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